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Research paper

Application of phospho-CyTOF to characterize immune activation in patients with sickle cell disease in an ex vivo model of thrombosis

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ABSTRACT

Sickle cell disease (SCD) is a genetic disease caused by mutations in the beta globin gene, and inflammation plays a key role in driving many aspects of disease pathology. Early immune activation is believed to be associated with hemodynamic stresses and thrombus formation as cells traffic through blood vessels. We applied an extracorporeal perfusion system to model these effects ex vivo, and combined this with a phospho-CyTOF workflow to comprehensively evaluate single-cell signatures of early activation across all major circulating immune subsets. These approaches showed immune activation following passage through the perfusion chamber, most notably in monocytes, which exhibited platelet aggregation and significantly elevated expression of multiple phospho-proteins. Overall, these studies outline a robust and broadly applicable workflow to leverage phospho-CyTOF to characterize immune activation in response to ex vivo or in vivo perturbations and may facilitate identification of novel therapeutic targets in SCD and other inflammatory diseases.

1. Introduction

Sickle cell disease (SCD) is a recessively inherited disorder caused by mutations of the beta globin gene. In the most severe form of SCD, Sickle Cell Anemia, both beta globin genes carry the glu-val substitution at codon 6 of the beta globin locus causing hemoglobin S to be produced exclusively instead of the wild-type hemoglobin A1. Hemoglobin S polymerizes when deoxygenated, forming long rods which damage the red cell membrane causing chronic hemolysis, reduced red cell lifespan and ultimately the clinical manifestations of SCD (most notably vaso-occlusion and organ damage) (Ballas, 2002; Walmet et al., 2003; Blann et al., 2003; Hebbel et al., 2004). While membrane damage by hemoglobin S polymers is the proximal event in SCD pathophysiology, inflammation is a key component in the development of vessel occlusion, thrombosis, and organ injury. Broad anti-inflammatory therapies, glucocorticoids, relieve SCD symptoms but precipitate severe rebound effects when discontinued and have substantial side effects (Strouse et al., 2008; Kumar et al., 2010; Quinn et al., 2011). Targeted anti-inflammatory therapies such as selectin inhibitors have shown more promise, but more research is needed to understand the role of inflammation in SCD to identify other targets of intervention.

Vaso-occlusion, the critical event which leads to the complications of SCD (including but not limited to stroke, pain and organ damage), is driven by two synergistic, interacting processes: inflammation and thrombosis. With hemoglobin S polymerization and red cell membrane damage as the inciting event, several downstream pathways become activated including "oxidative stress, nitric oxide (NO) depletion, endothelial dysfunction," (Torres et al., 2016) inflammation and thrombosis. Inflammatory cytokines including endothelin1, p and e-selectins, and soluble vascular cell adhesion molecule are elevated in SCD and levels correlate directly with SCD morbidity (Duits et al., 1996; Saleh et al., 1999; Duits et al., 2003; Schnog et al., 2003). Monocytes, neutrophils and iNKT cells have been implicated as key cell populations in mediating SCD vaso-occlusion (Field & Nathan, 2011; Frenette, 2002;

Abbreviations: SCD, Sickle cell disease; TEM, ThromboElastoMetry; exTEM, extrinsic TEM; inTEM, intrinsic TEM

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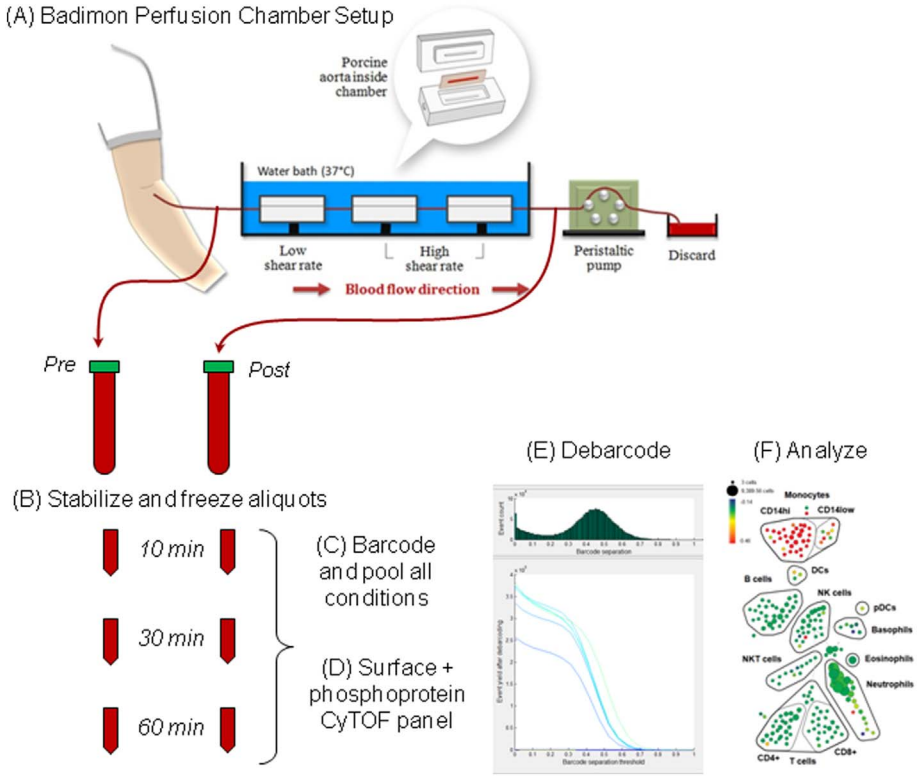

Fig. 1. Schematic of Badimon Vortex – Phospho-CyTOF experiment. An intravenous catheter was introduced and blood was drawn through a Badimon Perfusion Chamber using a peristaltic pump. Blood samples were collected before and after passing through the chamber in lithium heparin vacutainer tubes and incubated at room temperature. Aliquots were removed at 10, 30 or 60 min intervals and stabilized and cyropreserved using SmartTube buffer. Batches of samples were thawed, barcoded and processed for phospho-CyTOF analysis.

Chang et al., 2008; Hebbel et al., 1985). Additionally there are abnormalities at almost every level of the hemostatic system in SCD (SCD is one of few conditions which manifests both arterial and venous thrombosis including small, medium and large vessels) with several potential points of interaction between hemostasis and immune activation. These abnormalities (including platelet activation, abnormal thrombin kinetics and fibrinolysis) appear to be caused by and to further amplify inflammation and vaso-occlusion in SCD (Lim et al., 2013). The diffuse nature of the activation of inflammation and thrombosis in SCD (often referred to as “inflammatory soup”) is a barrier to identifying appropriate points for therapeutic intervention. Studies that identify the proximal steps of inflammatory and hemostatic activation may help to identify promising therapeutic targets.

By allowing the measurement of over 40 parameters in a single sample, mass cytometry (CyTOF) offers a powerful approach to dissect the phenotypic and functional heterogeneity of complex cell samples. In addition to allowing detailed characterization of cell populations on the basis of surface receptor expression patterns, CyTOF can also be used to evaluate signaling pathways using antibodies targeting phosophorylated protein epitopes (Bendall et al., 2011). This approach, referred to as phospho-CyTOF, can offer a detailed dynamic characterization of the nature of immune activation. In the current study, we describe the application of phospho-CyTOF to human whole blood samples characterize early immune activation events in associated with shearing and thrombotic stimuli using an ex vivo model of thrombosis. These analyses may be valuable in better delineating inflammatory pathways underlying SCD pathophysiology, and may offer a means to identify therapeutic targets in SCD and other diseases. We believe that the methods described here can also be broadly adapted to perform detailed analyses of early immune activation in a wide range of experimental and clinical settings.

2. Material and methods

2.1. Subjects and samples

The following study was approved by the Mount Sinai Institutional Review Board and written informed consent was obtained from all participants. This was a prospective experiment performed on 5 individuals with SCD. Two control cohorts were used; 23 age, gender and race matched individuals served as historical controls for clotting analyses and 4 unselected healthy donors served as controls for the immune activation CyTOF assays. The inclusion criteria were age > 18, confirmation of SCD status by hemoglobin electrophoresis. Individuals who were taking oral anticoagulants or antiplatelet medications were excluded, and individuals taking hydroxyurea were excluded. The median age of SCD patients was 28 (range 26–32) and the median age of controls was 57 (range 47–65) (Table 3). Racial characteristics were different between SCD patients and healthy controls in the CyTOF experiments, with the controls being predominantly White and individuals with SCD being predominantly Black.

On the day of the study patients reported to the AtheroThrombosis Research Unit of Mount Sinai. Following placement of a peripheral intravenous catheter, blood samples for CyTOF studies were collected and study to assess thrombus formation (described below) was performed. The IV catheter was then removed followed by 30 min of clinical observation to ensure that there were no procedural complications.

2.2. Ex vivo chamber assessment of thrombus formation

Thrombus formation was assessed using the Badimon Perfusion Chamber, an ex vivo model of thrombosis that measures the acutely formed, platelet-rich thrombus (Zafar et al., 2007). This model consists of three chambers in series, each containing a piece of porcine aorta stripped of the intimal layer. The shear rate of the first chamber was set at 212 s^{1} while the remaining two were 1,690 s^{1}, mimicking venous and moderately stenotic arterial flow conditions, respectively. A peristaltic

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